Methanofollis formosanus sp. nov., isolated from a fish pond

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A mesophilic, hydrogenotrophic methanogen, strain ML15^T, was isolated from an aquaculture fish pond near Wang-gong, Taiwan. The cells were irregular cocci, non-motile, 1.5–2.0 µm in diameter and Gram-negative. Cells of strain ML15^T lysed easily in the presence of SDS (0.1 g l⁻¹) and the S-layer protein had an *M_r* of 138 800. The catabolic substrates utilized by this strain included formate and H₂/CO₂, but not acetate, methanol, trimethylamine or secondary alcohols. Growth did not occur in minimal medium, but was observed when yeast extract and tryptone were added. Strain ML15^T grew fastest at 37 °C, pH 6.6–7.0 and with 3% NaCl. Acetate was not required for cell growth. Trace amounts of tungstate promoted cell growth. The G + C contents of DNA of *Methanofollis aquaemaris* N2F9704^T and strain ML15^T were 59.1 and 58.4 mol%, respectively. Sequence analysis of the 16S rRNA genes of strain ML15^T and selected *Methanofollis* species revealed similarities of 95–97%. Based on the data presented here, it is proposed that strain ML15^T (= OCM 789^T = DSM 15483^T) represents a novel species, *Methanofollis formosanus* sp. nov.

Based on results of DNA–DNA hybridization and 16S rRNA gene sequence analysis, hydrogenotrophic, irregularly coccoid methanogens of the family *Methanomicrobiaceae* have been separated into five genera: *Methanomicrobium*, *Methanogenium*, *Methanoculleus*, *Methanolacinia* and *Methanofollis* (Bleicher *et al.*, 1989; Blotvogel *et al.*, 1991; Boone *et al.*, 1993; Corder *et al.*, 1983; Jain *et al.*, 1988; Lai & Chen, 2001; Maestrojuán *et al.*, 1990; Ollivier *et al.*, 1986, 1998; Rivard & Smith, 1982; Romesser *et al.*, 1979; Rouvière *et al.*, 1992; Widdel, 1986; Widdel *et al.*, 1988; Xun *et al.*, 1989; Zellner *et al.*, 1997, 1998, 1999).

The genus *Methanofollis*, which includes *Methanofollis tationis* Chile 9^T and *Methanofollis liminatans* strains GKZPZ^T and BM1, was formally proposed by Zellner *et al.* (1999) on the basis of 16S rRNA gene sequencing and chemotaxonomic and physiological data. *Methanofollis tationis* Chile 9^T was isolated from a solfataric field on Mount Tatio in the Atacama Desert in northern Chile (Zabel *et al.*, 1984), whereas *Methanofollis liminatans* GKZPZ^T and BM1 were isolated from an industrial wastewater reactor in Germany (Zellner *et al.*, 1990,

1999). Recently, a novel methanogen isolated from an aquaculture fish pond near Wang-gong, Taiwan, was reported as *Methanofollis aquaemaris* N2F9704^T (Lai & Chen, 2001). These three *Methanofollis* species are all mesophilic, highly irregular cocci that use H₂/CO₂ and formate for growth and methanogenesis. However, *Methanofollis aquaemaris* N2F9704^T differs from the other strains in its source, substrate utilization, *M_r* of the S-layer protein subunit, effect of tungsten on growth and optimal ranges of NaCl and pH for growth. Moreover, the 16S rRNA gene sequence similarities of *Methanofollis aquaemaris* N2F9704^T with the other *Methanofollis* species were 94.7–95.5% (Lai & Chen, 2001). In this report, the isolation and characterization of a novel methanogen, strain ML15^T, which was isolated from an aquaculture fish pond in Taiwan, are described; it is proposed that this strain represents a novel species, *Methanofollis formosanus* sp. nov.

Strain ML15^T was isolated from a water sample of a marine aquaculture fish pond near Wang-gong, Taiwan, the same sampling site used for isolation of *Methanofollis aquaemaris* N2F9704^T (Lai & Chen, 2001). Sea water and ground water were mixed and pumped into a man-made pond to obtain 10–20‰ practical salinity units for the mixed cultivation of *Chanos chanos* (white mullet) and *Meretrix lusoria* (Lai *et al.*, 1999). Sampling, enrichment and isolation methods for strain ML15^T were according to Lai & Chen (2001). The modified anaerobic technique of Hungate was performed (Balch *et al.*, 1979; Sowers & Noll, 1995) and sterilized media were prepared under an oxygen-free N₂:CO₂ (4:1) atmosphere. The composition and preparation of MB medium

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ML15^T is AY186542.

An EM of strain ML15^T and graphs showing the effects of temperature, pH and salinity on growth of strain ML15^T are available as supplementary material in IJSEM Online.

was as described previously (Lai & Chen, 2001). MB/W medium was MB medium with tungstate (Na_2WO_4 , 0.3 mg l^{-1}). Minimal medium (MM) was MB medium without the addition of yeast extract and tryptone.

Enrichment was begun immediately after the sample was brought to the laboratory. Strain ML15^T was isolated and purified as described previously (Lai & Chen, 2001; Lai *et al.*, 2002, 2004). Samples (5 ml) from sediments of the marine water aquaculture fish pond were inoculated into a bottle of MB medium (45 ml) containing methanol as methanogenic substrate. The production of methane was determined by GC with flame-ionization detection (Lai *et al.*, 1999). After 1 month incubation at room temperature, methanogenesis occurred and, after four successive transfers, the methanogenic culture was then inoculated into roll-tube MB/W agar medium for further isolation. Under the fluorescent microscope, a fluorescence-positive colony with a small opaque centre and irregular translucent margin was picked and transferred to 5 ml MB/W medium with methanol in the Coy anaerobic chamber. The culture grew poorly, but good growth was observed after it was further transferred to MB/W medium with formate as substrate. Methane-producing cultures from this single colony were further purified by combining the serial dilution method (10^{-4}), antibiotic tetracycline ($100 \mu\text{g ml}^{-1}$) and roll tube method until contamination by non-methanogens was not detectable. This isolate, strain ML15^T, was previously known as N2M9705.

Cells of strain ML15^T were non-motile, irregular cocci, $1.5\text{--}2.0 \mu\text{m}$ in diameter and stained Gram-negative. Under the phase-contrast microscope (Olympus BH-2), the irregular coccus cells appeared with a dark centre surrounded by a transparent outer layer. Refractive areas were observed in the cells. As observed by SEM ABT-150S (sample preparation as described by Lai *et al.*, 1999), strain ML15^T presented irregular flat, disc-shaped cells with concavity (Fig. 1) and planes of division were frequently detected. Cells lysed rapidly in the presence of SDS (0.1 g l^{-1}), indicating that the cell envelope consisted of a protein surface layer (Boone & Whitman, 1988). The proteinaceous cell wall structure of strain ML15^T was very sensitive to physical forces, such as those exerted by centrifugation and pipetting, and lysed easily. Negative staining of strain ML15^T was performed as described previously (Lai & Shih, 2001) and cells observed under TEM (JEM-200cx; JEOL) showed a hexagonally arranged pattern of S-layer protein (see Supplementary Fig. A available in IJSEM Online). The centre-to-centre spacing of the morphological units of the S-layer lattice of strain ML15^T was about 16.4 nm . Surface-layer proteins were isolated according to the protocol of König (1995). SDS-PAGE was performed as described by Laemmli (1970) and Coomassie blue R-250 was used to visualize protein. Analysis of the S-layer indicated it was composed of a protein subunit with an M_r of 138 800.

Strain ML15^T is a strictly anaerobic organism; no growth

Fig. 1. SEM of strain ML15^T showing the disc-shaped irregular coccoid cells. Arrows show the division plane of cells. Bar, $1.0 \mu\text{m}$.

was observed at low oxygen levels. Under N_2/CO_2 atmospheres, strain ML15^T grew in MB/W medium supplemented with H_2 (100 %, 200 kPa) or sodium formate (100 mM), but not in MB/W medium supplemented with sodium acetate (50 mM), trimethylamine (40 mM), methanol (50 mM), ethanol (48 mM), 2-propanol (48 mM), iso-butanol (48 mM), 2-butanol (48 mM) or dimethylamine (40 mM). Specific growth rates were calculated from methane production, which was analysed by linear regression of the logarithm of the total amount of methane that accumulated over time. Inocula were grown under conditions similar to the experimental conditions. The specific growth rate of strain ML15^T in MB/W medium (containing yeast extract, tryptone and tungsten) with formate (50 mM) plus acetate (20 mM) was 0.019 h^{-1} (generation time 36 h). However, no growth was observed in MM/W, thus showing the heterotrophic nature of strain ML15^T. Addition of acetate (20 mM) shortened the lag period, but did not affect the specific growth rate, indicating that acetate was not required for growth of strain ML15^T. Addition of tungsten greatly promoted the cell growth rate, from 0.0096 h^{-1} in MB to 0.0191 h^{-1} in tungsten-containing MB/W medium.

The optimal growth parameters of strain ML15^T were tested using cells grown in MB/W medium with formate plus acetate. Strain ML15^T could grow at $25\text{--}42^\circ\text{C}$ and optimal growth was observed at 40°C . Temperatures below 20°C or above 50°C completely inhibited growth of cells (Supplementary Fig. B). Strain ML15^T grew at pH $5.6\text{--}7.3$, with optimal growth at pH $6.6\text{--}7.0$ (Supplementary Fig. B). At pH values greater than 7.7 , cell growth was completely inhibited suggesting that cells are sensitive to an alkaline environment. Cells of strain ML15^T could tolerate NaCl concentrations of $0\text{--}4\%$, but not above 6% . The optimal NaCl concentration for growth of strain ML15^T was 3% , with a doubling time of 20 h (Supplementary Fig. B).

The sensitivity of strain ML15^T to ampicillin, penicillin G, spectinomycin, kanamycin, tetracycline and chloramphenicol (each at a concentration of 100 µg ml⁻¹) was tested in MB/W medium with sodium formate (100 mM) plus acetate (20 mM) and 0.5 % NaCl at 37 °C. Results indicated that strain ML15^T was resistant to ampicillin, penicillin, kanamycin and spectinomycin and sensitive to chloramphenicol; tetracycline inhibited cell growth.

The melting temperatures of DNA from *Methanofollis aquaemaris* N2F9704^T and strain ML15^T were obtained as described previously (Jan *et al.*, 1999; Lai *et al.*, 2004; Marmur & Doty, 1962) and were 93.52 and 93.25 °C, respectively. The DNA G+C contents of *Methanofollis aquaemaris* N2F9704^T and strain ML15^T were 59.07 and 58.41 mol%, respectively.

For phylogenetic analysis, DNA was isolated by the general procedure of Jarrell *et al.* (1992). Approximately 30 ng DNA was used as a template for PCR amplification of an approximately 1300 bp portion of the 16S rRNA gene. PCR amplification primers used were forward primer coccus 1 (5'-CGACTAAGCCATGCGAGTC-3') and reverse primer reverse 3 (5'-GTGACGGGCGGTGTGTGCAAG-3'). The sequences were checked by the program CHECK-PROBE from the Ribosomal Database Project (Maidak *et al.*, 1996) and corresponded to positions 23–41 and 1327–1309 in the 16S rRNA nucleotide sequence of *Methanofollis aquaemaris* N2F9704^T (AF262035). As well as the two amplification primers, two additional primers were used for sequencing: shih2 (5'-CGATTACAGGGTTTCACTCCTACC-3') and primer follis1 (5'-TAATCGGTACGGGTTGTG-3') (*Methanofollis aquaemaris* N2F9704^T, accession number AF262035, sequence positions 506–482 and 124–141, respectively). The resulting sequence of strain ML15^T was assembled to produce an approximately 1287 base contiguous rRNA gene sequence (positions 22–1308). Gene sequences of the archaea used were obtained from the Ribosomal Database Project and GenBank. The similarity matrix was obtained based on the analytical results of the Ribosomal Database Project (<http://rdp.cme.msu.edu/html/>). Multiple sequence alignments were analysed using the package CLUSTAL_W at the Biology Workbench (<http://workbench.sdsc.edu/>). Distances were computed with the package CLUSTAL TREE at the same website using the neighbour-joining model and fed to DRAWGRAM in the program package PHYLIP version 3.5c (Felsenstein, 1993). Bootstrap confidence analysis was performed with SEQBOOT of the PHYLIP package using 1000 replicates.

The 16S rRNA gene sequence (1287 nt) of strain ML15^T was determined and phylogenetic trees were constructed using a selection of sequences from related methanogens obtained from the GenBank database (Fig. 2). 16S rRNA gene sequence similarities between strain ML15^T and other members of the families *Methanocorpusculaceae*, *Methanomicrobiaceae* and *Methanoplanaceae* were 85, 91–96 and 91 %, respectively. 16S rRNA gene sequence analysis placed strain ML15^T close to *Methanofollis aquaemaris* N2F9704^T

Fig. 2. Phylogenetic analysis of 16S rRNA gene sequences showing the relationship between strain ML15^T and various methanogens. The significance of each branch is indicated by bootstrap percentages. Accession numbers for reference species are shown in parentheses. Bar, 0.02 evolutionary distance.

(sequence similarity 97.2 %), *Methanofollis liminatans* GKZPZ^T (95.5 %) and *Methanofollis tationis* Chile 9^T (96.3 %).

Strain ML15^T belongs to the archaeal domain on the basis of envelope composition, metabolism, resistance to antibiotics and 16S rRNA gene sequence (Woese *et al.*, 1990). Devereux *et al.* (1996) and Fry *et al.* (1991) have proposed that a similarity of less than 98 % in a 16S rRNA sequence is evidence for separate species. The closest relative to strain ML15^T was *Methanofollis aquaemaris* strain N2F9704^T at 97.2 % similarity; *Methanofollis liminatans* GKZPZ^T (sequence similarity 95.5 %) and *Methanofollis tationis* Chile 9^T (sequence similarity 96.3 %) were also closely related. The 16S rRNA gene sequence similarity of strain ML15^T to the other members of the *Methanomicrobiales* was 85–93 %. Characteristics that differentiate strain ML15^T from related *Methanofollis* species are listed in Table 1. These phylogenetic, phenotypic and physiological distinctions indicate that strain ML15^T represents a novel *Methanofollis* species, for which the name *Methanofollis formosanus* sp. nov. is proposed.

The genus *Methanofollis* includes *Methanofollis tationis* Chile 9^T (=DSM 2702^T), *Methanofollis liminatans* strains GKZPZ^T (=DSM 4140^T) and BM1 (=DSM 10196) (Zellner *et al.*, 1999) and *Methanofollis aquaemaris* strain N2F9704^T (Lai & Chen, 2001). All these organisms are mesophilic, highly irregularly cocci that use H₂/CO₂ and formate for growth and methanogenesis. Although strain ML15^T shares these traits with *Methanofollis* species, it differs from these species in its source, mobility, substrate utilization and requirements, halotolerance, alkali tolerance, *M_r* of S-layer protein subunits, growth in the presence of tungsten and phylogenetics (Table 1). Strain ML15^T and *Methanofollis aquaemaris* strain N2F9704^T were isolated

Table 1. Characteristics of *Methanofollis* species and strain ML15^T

Taxa: 1, strain ML15^T (data from this study); 2, *M. aquaemaris* N2F9704^T (Lai & Chen, 2001); 3, *M. tationis* Chile 9^T (Zabel *et al.*, 1984); 4, *M. liminatans* GKZPZ^T (Zellner *et al.*, 1990); 5, *M. liminatans* BM1 (Zellner *et al.*, 1999). ND, Not determined.

Characteristic	1	2	3	4	5
Cell size (µm)	1.5–2.0	1.2–2.0	1.5–3.0	1.25–2.0	1.25–2.0
Flagella	—	—	+	+	ND
S-layer subunit M_r ($\times 10^{-3}$)	138.8	137	120	118	ND
Lattice constant c/c (nm)*	16.4	11	ND	15.4	ND
Substrates†	H, F	H, F	H, F	H, F, 2P, 2B	H, F, 2P, 2B
Temperature for growth (°C):					
Optimum	40	37	37	40	40
Range	20–42	20–43	25–45	25–45	20–45
Optimum pH	6.6	6.5	7.0	7.0	7.0
NaCl concentration for growth (%):					
Optimum	3	0.5	0.8–1.2	ND	ND
Range	0–6	0–6	0–7	0–2.9	ND
Tungsten requirement‡	S	S	R	S	S
Acetate requirement‡	S	S	R	R	R
G + C content (mol%)	58.4	59.1	54.0	59.3	60.0
Source	Aquaculture fish pond	Aquaculture fish pond	Solfataric field	Wastewater reactor	Wastewater reactor

*Centre-to-centre spacing of hexagonally arranged protein subunits of the S-layer.

†H, H₂/CO₂; F, formate; 2P, 2-propanol/CO₂; 2B, 2-butanol/CO₂.

‡R, Required; S, growth stimulated.

from the same source (aquaculture fish pond) and, morphologically, they are very similar, non-motile irregular cocci, unlike *Methanofollis tationis* and *Methanofollis liminatans*, which were motile with flagella. Cells of members of the family *Methanomicrobiaceae* have hexagonal S-layer lattices consisting of glycoprotein subunits with M_r of 101 000–155 000 (Zellner *et al.*, 1999). All *Methanofollis* species possess surface layer proteins as a cell envelope, forming a soft bag for the cells. The M_r of the S-layer protein subunits from ML15^T and *Methanofollis aquaemaris* strain N2F9704^T were 138 800 and 137 000, respectively; M_r of the S-layer protein subunits for *Methanofollis tationis* and *Methanofollis liminatans* were around 120 000. Also, both strain ML15^T and *Methanofollis aquaemaris* strain N2F9704^T could only use formate and H₂/CO₂ as catabolic substrates, could not utilize 2-propanol/CO₂ or 2-butanol/CO₂ and did not require acetate for cell growth, although it could reduce the lag time. In contrast, both *Methanofollis tationis* and *Methanofollis liminatans* required acetate for cell growth and *M. liminatans* could use 2-propanol/CO₂ and 2-butanol/CO₂ to produce methane.

Within the order *Methanomicrobiales*, the growth requirement for tungsten has been reported in *Methanoculleus palmolei* (Zellner *et al.*, 1998) and *Methanofollis tationis* (Zabel *et al.*, 1984). No growth was observed in *Methanofollis tationis*, an organism isolated from a solfataric field, if tungsten was not present in the growth medium (Zabel

et al., 1984). In contrast, growth of the related organisms *Methanofollis liminatans*, isolated from effluent of a wastewater reactor (Zellner *et al.*, 1990, 1999), *Methanocalculus chunghsingensis*, from an estuarine environment (Lai *et al.*, 2002), and *Methanofollis aquaemaris* and strain ML15^T, from an aquaculture fish pond (Lai *et al.*, 2004 and this study), was greatly stimulated by tungsten, but the element was not absolutely required for growth. In the case of the estuarine isolate *Methanocalculus taiwanensis* P2F9704a^T, the trace element tungsten was not required, but it slightly stimulated growth and could extend the growth range relating to temperature, pH and salt concentration (Lai & Chen, 2001).

All known *Methanofollis* species are mesophilic and neutrophilic. However, the pH range for growth of strain ML15^T was pH 5.6–7.3 and cell growth was completely inhibited at pH 7.7, indicating that this strain is more sensitive to an alkaline environment than other species. Also, the optimal salt (NaCl) concentration for growth of strain ML15^T was 3%, which is the highest concentration observed in *Methanofollis* species. Phylogenetic (Fig. 2), phenotypic and physiological distinctions (Table 1) between *Methanofollis* species suggest that these species form two clusters, with *M. aquaemaris* and strain ML15^T constituting one cluster. The 16S rRNA gene sequence similarities of *Methanofollis aquaemaris* and strain ML15^T to *Methanofollis liminatans* GKZPZ^T were within the range 95.3–96.3%.

Description of *Methanofollis formosanus* sp. nov.

Methanofollis formosanus (for.mo.sa'nus. N.L. masc. adj. *formosanus* from Formosa, the beautiful island of Taiwan).

Irregularly coccoid cells, non-motile, 1.5–2.0 µm in diameter. Obligately anaerobic cells. Stains Gram-negative. Cell wall has an SDS-sensitive S-layer protein with an M_r of 138 800. Catabolic substrates used include H_2/CO_2 and formate, but not acetate, methanol, trimethylamine, dimethylamine, ethanol, 2-propanol, iso-butanol, 2-butanol or dimethylamine. Cells are mesophilic and grow at 20–42 °C, with optimal growth at 37 °C. Cells grow at pH 5.6–7.3, with optimal growth at pH 6.6. Cells grow well in 0–4 % NaCl, with optimal growth at 3 % NaCl. Addition of acetate reduces the lag time and the trace element tungsten greatly promotes cell growth and extends the growth range. No growth is detected in minimal medium. Growth is completely inhibited by chloramphenicol and partly inhibited by tetracycline, but not by ampicillin, penicillin, kanamycin or spectinomycin. The G+C content of DNA of strain ML15^T is 58.4 mol%.

The type strain is strain ML15^T (=OCM 789^T=DSM 15483^T), isolated from a marine water aquaculture fish pond near Wang-gong, Taiwan.

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